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FEBS Letters 582 (2008) 1182–1188

Phosphoregulation of MgcRacGAP in mitosis involves Aurora B and Cdk1 protein kinases and the PP2A phosphatase

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Received 17 July 2007; revised 29 November 2007; accepted 7 December 2007

Available online 15 January 2008

Edited by Angel Nebreda

Abstract MgcRacGAP, a Rho GAP essential to cytokinesis, works both as a Rho GTPase regulator and as a scaffolding protein. MgcRacGAP interacts with MKLP1 to form the centralspindlin complex and associates with the RhoGEF Ect2. The GAP activity of MgcRacGAP is regulated by Aurora B phosphorylation. We have isolated B56ε, a PP2A regulatory subunit, as a new MgcRacGAP partner. We report here that (i) MgcRacGAP is phosphorylated by Aurora B and Cdk1, (ii) PP2A dephosphorylates Aurora B and Cdk1 phosphorylated sites and (iii) inhibition of PP2A abrogates MgcRacGAP/Ect2 interaction. Therefore, PP2A may regulate cytokinesis by dephosphorylating MgcRacGAP and its interacting partners.

Structured summary:

- MINT-6166761: *Aurora B* (uniprotkb:Q96GD4) *phosphorylates* (MI:0217) *MgcRacGAP* (uniprotkb: Q9H0H5) by *protein kinase assay* (MI:0424)
- MINT-6166774: *Cdk1* (uniprotkb:P06493) *phosphorylates* (MI:0217) *MgcRacGAP* (uniprotkb:Q9H0H5) by *protein kinase assay* (MI:0424)
- MINT-6166653: *PP2A* (uniprotkb:P67775) *dephosphorylates* (MI:0203) *MgcRacGAP* (uniprotkb:Q9H0H5) by *phosphatase assay* (MI:0434)
- MINT-6166710, MINT-6166727, MINT-6166735: *MgcRacGAP* (uniprotkb:Q9H0H5) *physically interacts* (MI:0218) with *B56ε* (uniprotkb:Q16537) by *coimmunoprecipitation* (MI:0019)
- MINT-6166691: *B56ε* (uniprotkb:Q16537) *physically interacts* (MI:0218) with *MgcRacGAP* (uniprotkb:Q9H0H5) by far *western blotting* (MI:0047)
- MINT-6166556: *MgcRacGAP* (uniprotkb:Q9H0H5) *physically interacts* (MI:0218) with *B56ε* (uniprotkb:Q16537) by *two-hybrid* (MI:0018)
- MINT-6166634: *MgcRacGAP* (uniprotkb:Q9H0H5) *physically interacts* (MI:0218) with *Ect2* (uniprotkb:Q9H8V3) by *coimmunoprecipitation* (MI:0019)
- MINT-6166596: *MKLP-1* (uniprotkb:Q02241) *physically interacts* (MI:0218) with *B56ε* (uniprotkb:Q16537) , *MgcRacGAP* (uniprotkb:Q9H0H5), *PP2A* (uniprotkb:P67775) by *coimmunoprecipitation* (MI:0019)

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Keywords: RhoGAP; Phosphorylation; Phosphoprotein Phosphatase 2A; Cytokinesis

1. Introduction

Rho family GTPases are major regulators of actin polymerization and microtubule stabilization [1,2] and thereby control many aspects of mitosis. During cytokinesis, induction and progression of the contractile ring depend on RhoA activation [3], whereas Rac activity is down regulated [4]. Recently, Cdc42 has been proposed to work in metaphase by regulating attachment of spindle microtubules to kinetochores [5,6].

Two Rho regulatory proteins, i.e. Ect2 and MgcRacGAP, are involved in many of these processes. Ect2 is a RhoGEF allowing RhoA activation in contractile ring formation [7,8], and Cdc42 activation in prometaphase [6]. MgcRacGAP is a Rac/Cdc42 GAP conserved from *C. Elegans* to mammals [9,10] which is required for cytokinesis, possibly through Rac activity suppression [4,11]. MgcRacGAP has also been shown to regulate Cdc42 during metaphase [6,12]. Both Ect2 and MgcRacGAP localize in the nucleus of interphasic cells, associate to the spindle in metaphase and anaphase and accumulate at the midbody during cytokinesis [10,13]. MgcRacGAP

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doi:10.1016/j.febslet.2007.12.036

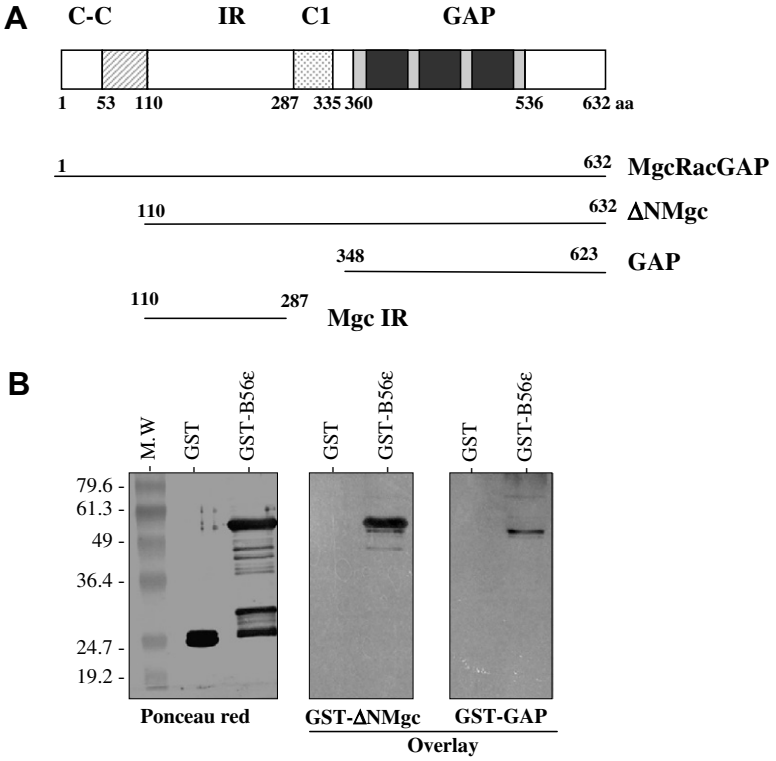

Fig. 1. MgcRacGAP interacts with B56ε in vitro. (A) Schematic representation of human MgcRacGAP domains and MgcRacGAP fragments used in this study. C–C: coiled-coil; C1: cysteine rich domain; IR: intermediate region; GAP: RhoGAP domain with three conserved blocks. MgcRacGAP: full length MgcRacGAP (aa 1–632); ΔNMgc: MgcRacGAP deleted of 109 Nterminal aminoacids (aa 110–632); GAP: fragment containing the RhoGAP domain (aa 348–623); MgcIR: intermediate region (aa 110–287) used as bait in two-hybrid screens. (B) Control GST and GST-B56ε fusion proteins were resolved on PAGE, electroblotted onto nitrocellulose filters and stained with Ponceau red (left panel). Filter immobilized proteins were probed with GST-ΔNMgc (middle panel) or GST-GAP (right panel); bound probes were revealed with an anti-MgcRacGAP antibody.

associates with the kinesin-like protein MKLP1, resulting in a heterotetrameric complex designated centralspindlin, which is required for microtubule bundling [14,15]. In addition, Ect2 directly binds to MgcRacGAP in the centralspindlin complex [15,16].

MgcRacGAP is phosphorylated at multiple sites by the mitotic kinase Aurora B. Hence, phosphorylation at Ser 387, in the GAP domain, has been proposed to convert MgcRacGAP to an active GAP towards RhoA [17]. In addition, the mitotic spindle associated protein PRC1 has been found to bind to MgcRacGAP and to inhibit its GAP activity towards Cdc42 during metaphase [6,12]. Interestingly, the phosphorylation of MgcRacGAP by Aurora B in the intermediate region (MgcIR aa: 110–287; Fig. 1A) might prevent PRC1 binding and release Cdc42GAP inhibition. Finally, recent reports indicate that the mitotic kinase Cdk1 phosphorylates Ect2 and thereby regulates its GEF activity and its interaction with MgcRacGAP [16,18].

Overall, these results point to the multiplicity and critical importance of phosphorylation events in regulating MgcRacGAP functions throughout mitosis.

In the present paper, we report that, in prometaphase-arrested T lymphocytes, MgcRacGAP is phosphorylated by Aurora B kinase, as previously observed, and by the mitotic kinase Cdk1. We also identify a novel player in the phosphoregulation of MgcRacGAP, by showing that protein phosphatase PP2A associates with the intermediate region of MgcRacGAP through B56 regulatory subunits and dephosphorylates MgcRacGAP at Aurora B and Cdk1 target sites.

2. Materials and methods

2.1. Reagents

The following chemicals were used: okadaic acid (Calbiochem), nocodazole and roscovitin (Sigma). Hesperadin was a kind gift from Dr. N. Kraut (Boehringer Ingelheim Austria GmbH).

Monoclonal antibodies against c-Myc-tag (mouse 9E10, Boehringer Mannheim), HA-tag (rat 3F10, Roche or mouse 16B12, Babco) and FLAG-tag (mouse M2, Sigma) were used in immunoprecipitation and immunoblotting experiments. In overlay assays, bound MgcRacGAP was detected with a polyclonal antibody to the GAP domain of human MgcRacGAP [9]. Goat polyclonal antisera to MgcRacGAP (ab2270) and to the B56 epsilon subunit (PPP2R5E) of PP2A were from abcam (Cambridge, UK). Anti phospho-TP antibody was from Cell Signaling Technology (New England Biolabs, Ipswich, MA) and anti-Ect2, anti-MKLP1 and anti-PP2A-cat from Santa Cruz (SantaCruz, CA). Active recombinant Aurora B and Cdk1 (provided as a complex with a GST-cyclin B fusion protein) were purchased from Upstate Biotechnology (Lake Placid, NY). Active PP2A was obtained from Promega France.

2.2. Constructs used in this study

cDNA constructs encoding various domains of MgcRacGAP (Fig. 1A) for expression in *Escherichia coli*, in mammalian cells and in yeast for two-hybrid cloning have been previously described [9,13,19,20]. A partial B56ε cDNA was inserted in a pGEX plasmid to produce a GST-B56ε (aa 132–435) fusion protein. Mutations of threonine residues into alanine were performed with oligonucleotides containing appropriate codon changes, using the Quikchange mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions.

2.3. Yeast two-hybrid cloning

The intermediate region (MgcIR) of MgcRacGAP (Fig. 1A) was fused to lexA in the pLex plasmid pVJL10 and used as a bait to screen a pACT2 human testis cDNA library (Clontech) according to published methods [19,21].

2.4. Overlay assay

Three micrograms of GST or six micrograms of GST-B56ε (aa 132–435) proteins were immobilised on nitrocellulose membrane according to standard procedures of Western blotting. The filter was then incubated with either GST-ΔNMgc (3.5 μg/mL) or GST-MgcGAP (2.6 μg/mL), for 1 h at room temperature, in PBS-Tween 0.1–5% milk. The filters were rinced in PBS-Tween 0.1% and immunodetection of the bound fraction of ΔNMgc or MgcGAP protein was performed using an antibody to the GAP domain [9].

2.5. Immunoprecipitation experiments in COS cells

COS cells were transiently transfected with the appropriate expression vectors using the FUGENE 6 reagent (Roche Molecular Biochemical). Twenty-four hours after transfection, cells were washed in cold PBS and proteins were extracted in the following lysis buffer: 50 mM Hepes pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1% Triton, 0.5% NP40, protease inhibitors. Cell lysates were incubated overnight at 4 °C, using anti-tag antibodies together with protein A-Sepharose CL-4B (Amersham). Beads were then washed in the lysis buffer and bound proteins analysed by Western blot using the appropriate antibody.

2.6. T-cell culture

The human T-cell leukemia-derived, IL-2-dependent Kit 225 cell line was maintained in RPMI 1640 culture medium with 10% fetal calf serum and 0.5 nM recombinant human IL-2 (Proleukin, Chiron Corp., The Netherlands).

Cells were synchronized by IL-2 deprivation, which arrests them in G1, and set back in culture with IL-2. After 24 h, nocodazole was added at a final concentration of 40 ng/mL for an additional 16 h. When indicated, okadaic acid (100 nM) was added for the last 5 h of the nocodazole block.

2.7. Phosphorylation/dephosphorylation in vitro

Affinity-purified GST-MgcRacGAP fusion proteins (2.5 μg) were incubated with 250 ng Cdk1 or 1 μg Aurora B in 30 μL of kinase buffer (MOPS 20 mM pH 7.2, β-glycerophosphate 20 mM, $MgCl_2$ 20 mM, DTT 2 mM, ATP 20 μM, PNPP 40 mM, EGTA 1 mM) supplemented with 10 μCi of [γ-^{32}P] ATP for 30 min at 30 °C. Fusion proteins were then adsorbed on GSH-agarose, washed three times in 1% triton X-100, Tris–NaCl buffer, and three more times in phosphatase buffer (Tris 50 mM pH 8.3, 20 mM $MgCl_2$, 1 mM DTT). One half of the beads were then incubated at 30 °C for 1 h with constant shaking in 30 μL phosphatase buffer containing two units of active PP2A. Following three washes, beads were boiled in 2X Laemmli sample buffer, proteins were resolved by SDS–PAGE and electrotransferred onto PVDF membranes (Amersham Biosciences). Signals from ^{32}P-labeled proteins were acquired quantitatively using a STORM® 480 phosphor-imager (Amersham Biosciences). Membranes were then processed for Western blot analysis.

2.8. Mass spectrometric analysis

GST-GAP was phosphorylated in vitro with Cdk1 as above, separated by SDS–PAGE and stained with colloidal coomassie blue. The protein in the gel piece was subjected to tryptic digestion as described previously [22]. Briefly, the gel was washed and dried in a Speed-Vac (Savant), the protein was reduced with β-mercaptoethanol and then modified with 4-vinylpyridine. The gel was then washed, dried and incubated with modified trypsin (Promega) overnight (18 h). The enzyme digest was removed from the gel, and the gel residue was extracted with 200 μL of 0.1% formic acid. These two fractions were combined, dried in a Speed-Vac, and kept at −20 °C for storage. The sample was resuspended in 0.1% formic acid for LC-MS/MS analysis. An aliquot of the sample was analyzed in a LC-MS/MS system consisting of Agilent 1200 nanoflow HPLC and LTQ-Orbitrap hybrid tandem mass spectrometer (ThermoFisher, USA). TurboSequest program was used to generate a list of unmodified peptides. The in-house program, *PTM Finder* (Tsay, manuscript in preparation), was used to automatically screen out the phosphopeptide candidates. Another aliquot of the remaining tryptic digest was analyzed by LC-MS/MS in the mass-specific mode for acquisition of the tandem mass spectra of these candidates. Annotation of the peak list in the MS/MS was facilitated by an in-house program.

3. Results and discussion

3.1. PP2A regulatory subunit B56 binds to MgcRacGAP

MgcRacGAP comprises several functionally distinct and conserved domains (Fig. 1A). Using MgcIR as a bait in

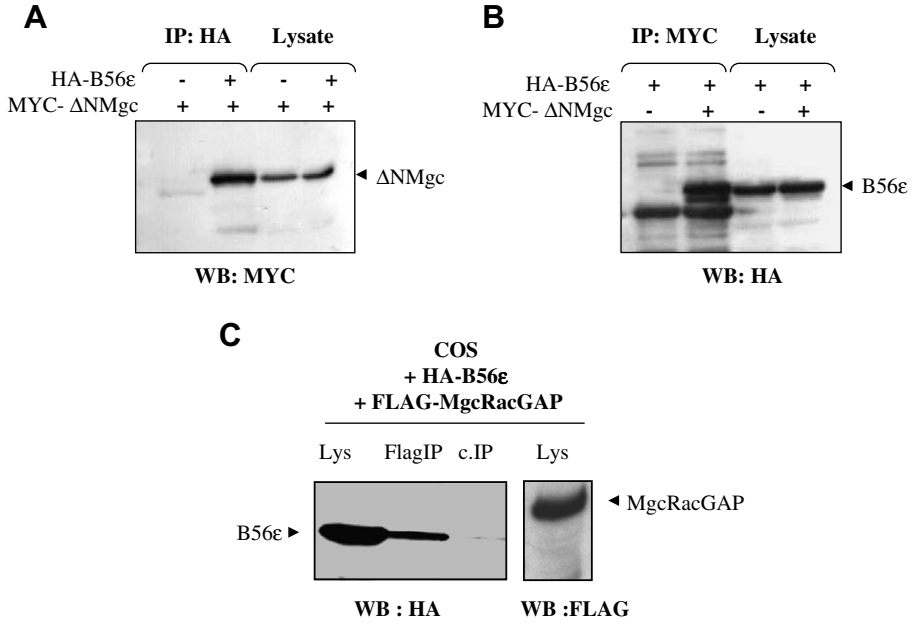

Fig. 2. Interaction between MgcRacGAP and B56 expressed in COS cells. (A) Coimmunoprecipitation (coIP) of B56ε and ΔNMgc. Lysates from COS cells expressing HAtagged B56ε and Myc-tagged ΔNMgc were immunoprecipitated with anti-HA antibodies followed by immunoblotting with anti-Myc antibodies. (B) Reciprocal immunoprecipitation using anti-Myc antibodies was revealed with anti-HA (mouse 16B12 antibody). (C) CoIP of B56ε and full length MgcRacGAP. Lysates from COS cells expressing HA-B56ε and Flag-MgcRacGAP were immunoprecipitated with anti-Flag antibodies, followed by immunoblotting with anti-HA (rat 3F10 antibody); c.IP (control IP) refers to a control experiment in which the immunoprecipitating antibody was omitted. The results shown here are representative of at least three independent experiments.

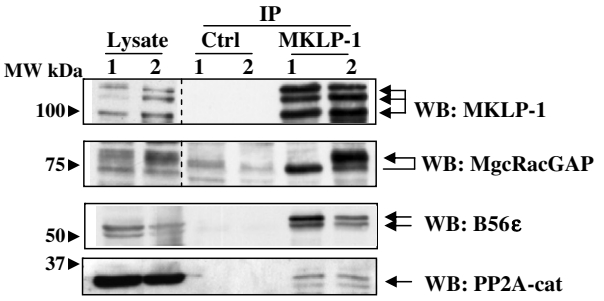

Fig. 3. B56ε and PP2A catalytic subunit co-precipitate with MgcRacGAP in Kit 225 cells. Lysates from S phase (1) or nocodazole-arrested cells (2) were immunoprecipitated with anti-MKLP1 antibodies and analyzed in Western blot (WB) with the indicated antibodies. Images in the upper two panels are reconstructed to show lysates in a slightly longer exposure than IPs. PP2A-cat, catalytic subunit of PP2A.

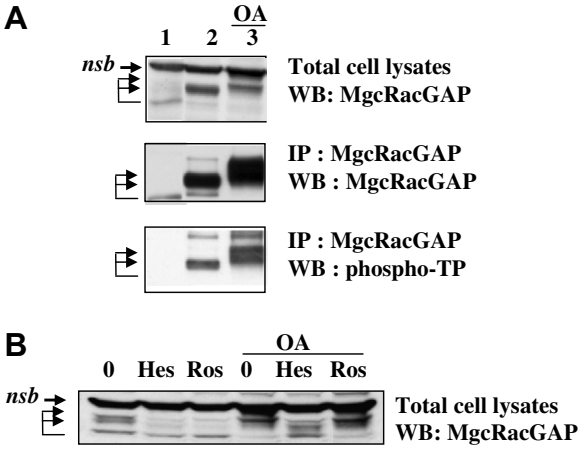

Fig. 4. MgcRacGAP phosphorylation is increased by okadaic acid and inhibited by roscovitin or hesperadin. (A) Cell lysates were prepared from Kit 225 cells synchronized in S phase (lane 1), cells arrested in prometaphase with nocodazole (lane 2), and nocodazole-arrested cells treated with okadaic acid (OA, lane 3) and immunoblotted with anti-MgcRacGAP antibodies (top panel). *nsb*, non-specific band. MgcRacGAp was immunoprecipitated from the above lysates and membranes were probed with anti- MgcRacGAP (middle panel) or anti-phospho-TP (lower panel) antibodies. (B) Nocodazole-arrested cells were treated with hesperadin (Hes, 2 μM) or roscovitin (Ros, 100 μM) together with or without OA, then lysed and analyzed in Western blot with anti-MgcRacGAP antibodies.

two-hybrid screens, we isolated several clones showing a perfect match with the cDNA sequence of B56ε (Accession No.:NP_006237.1 GI:5453956) a 467aa regulatory subunit of PP2A [23,24]. While full length MgcRacGAP could not be assayed due to expression failure in our two-hybrid system, we found that ΔNMgc (aa 110–632) exhibited specific interaction with B56ε clones in yeast and that, by contrast the GAP domain (aa 348–623) did not bind to B56ε (data not shown).

We next showed (Fig. 1B) that a filter immobilized GST-B56ε (aa 132–435) fusion protein can bind ΔNMgc but much less efficiently the GAP domain, thus indicating that MgcRacGAP associates with B56ε through a direct interaction involving MgcIR.

When expression vectors for ΔNMgc and HA-B56ε were co-transfected into COS cells, the two proteins could be readily coimmunoprecipitated indicating that they associate within the cell. Moreover, B56ε could be efficiently coimmunoprecipitated with full length MgcRacGAP (Fig. 2). B56 protein family consists of five isoforms (α, β, γ, δ, ε), all of which contain a conserved central region. We also observed that B56α can associate with ΔNMgc (Supplementary Fig. S1). Therefore, since B56α gene is known to be widely expressed [24], MgcRacGAP/B56 interaction may take place in many cell types.

3.2. PP2A regulatory subunit B56ε associates with endogenous MgcRacGAP in dividing T lymphocytes

Our recent observations indicate that MgcRacGAP expression peaks in G2/M in dividing T lymphocytes [25]; therefore, to confirm B56/MgcRacGAP association in a physiological setting, we turned to analyzing endogenous MgcRacGAP in the human Kit 225 T-cell line. Since MgcRacGAP has been assigned critical functions in the control of mitosis, we compared cells synchronized in S-phase to cells arrested in prometaphase with nocodazole. For unknown reasons, anti-MgcRacGAP antibodies failed to reproducibly co-immunoprecipitate B56ε (not shown). However, when MgcRacGAP was precipitated indirectly with anti-MKLP1 antibodies, a doublet at 55/56 kDa representing B56ε was detectable, indicating that B56ε was associated with centralspindlin in dividing T cells, presumably via its interaction with MgcRacGAP (Fig. 3). Not only B56ε, but also the catalytic subunit of PP2A was shown to co-precipitate with MgcRacGAP under these conditions. Interestingly, in prometaphase arrested cells, MgcRacGAP underwent a dramatic migration shift, highly suggestive of phosphorylation events as detailed below.

B56 regulatory subunits associate with a core dimer formed by a 65 kDa scaffolding subunit (A) and a 36 kDa catalytic subunit (PP2Ac) to generate the active trimeric PP2A holoenzyme, and thereby control the subcellular location and substrate specificity of PP2A. According to this scheme, active PP2A would be targeted to MgcRacGAP through B56 subunits to participate in MgcRacGAP phosphoregulation.

3.3. Phosphoregulation of MgcRacGAP in T lymphocytes

To further characterize MgcRacGAP phosphorylation in intact cells, Kit 225 cells were arrested in prometaphase and MgcRacGAP phosphorylation was estimated by the intensity of the shifted band and by its reactivity with an anti-phospho-TP antibody. To assess the role of PP2A, MgcRacGAP phosphorylation was analyzed in cells which have been pretreated or not with okadaic acid, a known inhibitor of PP2A. Of note, okadaic acid needs to be used at 100 nM to efficiently block PP2A in Kit 225 cells, a concentration which however does not affect PP1 as assessed on myosin light chain, a well characterized PP1 substrate (see Supplementary Fig. S2). As shown in Fig. 4A, MgcRacGAP immunoblots revealed at least three main detectable phosphorylation status: a presumably non-phosphorylated state in S-phase cells, a first level of phosphorylation in nocodazole-arrested cells and a complex hyperphosphorylated status induced by phosphatase inhibition. This pattern is compatible with multiple Aurora B dependent S/T phosphorylations. However, detection of phosphoTP is consistent with MgcRacGAP being also a substrate for a proline directed serine/threonine kinase. Indeed, upon treatment of Kit 225 cells with hesperadin or roscovitin, inhibitors of Aurora B kinase and Cdk1, respectively, Mgc-

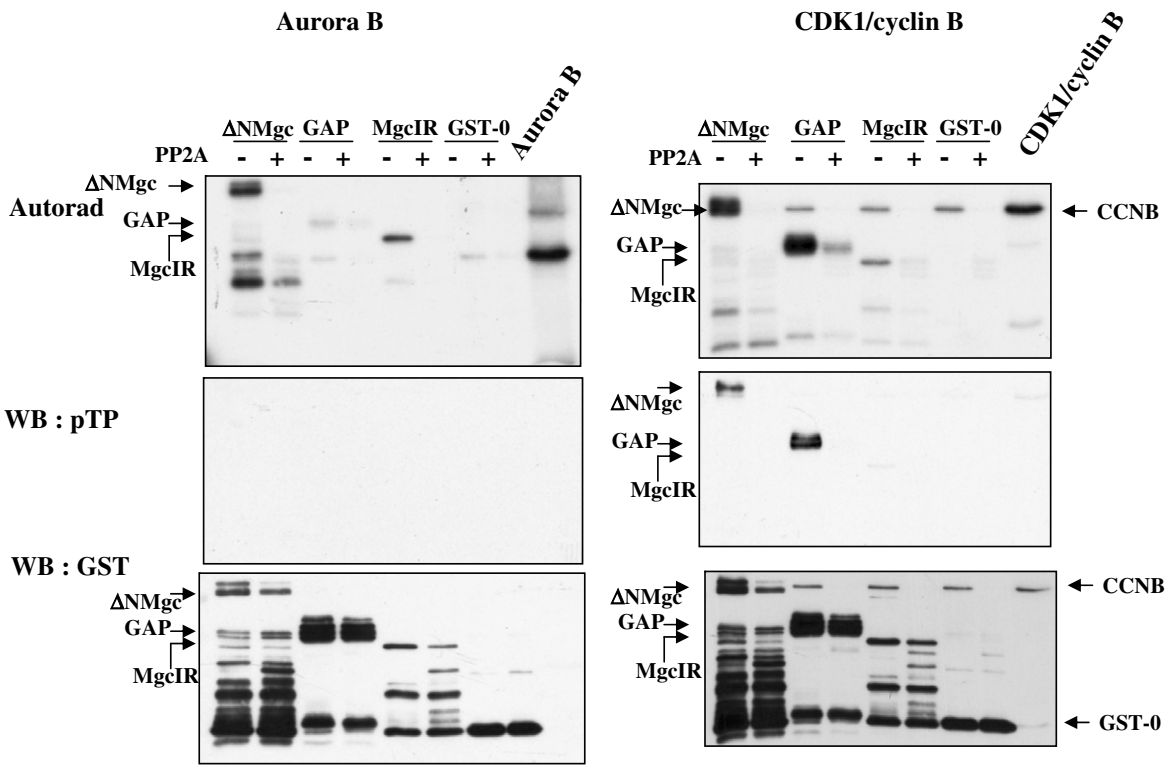

Fig. 5. MgcRacGAP phosphorylation in vitro by Aurora B and Cdk1 and dephosphorylation by PP2A. GST-ΔNMgc (aa 110–632), GST-MgcIR (aa 110–287), GST-GAP (aa 348–623) and GST-0 as a control were subjected to an in vitro kinase assay in the presence of [γ-^{32}P] ATP with recombinant Aurora B (left-hand side) or Cdk1 (right-hand side). One half of the reaction products were then further incubated with PP2A. The top panels show autoradiographs of the reaction products following SDS–PAGE and transfer to a PVDF membrane. Membranes were then blotted with anti-phospho-TP antiserum (middle panels) and anti GST-antibodies (bottom panels).

RacGAP phosphorylation was markedly inhibited (Fig. 4B), thus confirming that MgcRacGAP may be a substrate for both kinases. Furthermore, inhibition of Aurora B interferes with the supershift induced by okadaic acid.

3.4. *In vitro analysis of MgcRacGAP phosphorylation and dephosphorylation*

We then investigated MgcRacGAP phosphorylation by Aurora B and Cdk1 *in vitro*. Both Aurora B and Cdk1 efficiently phosphorylated MgcRacGAP (Fig. 5). Consistent with a previous report [12], Aurora B was found to phosphorylate primarily MgcIR (aa 110–287) whereas most of the signal induced by Cdk1 resided in the c-terminus part of the protein (aa 348–623). In addition, phosphorylation by Cdk1 resulted in MgcRacGAP acquiring immunoreactivity with an anti-phospho-TP antiserum.

In the same set of experiments, phosphorylated subdomains of MgcRacGAP were incubated in the presence of two units of active PP2A for 1 h at 30 ° C which resulted in nearly complete removal of the ^{32}P label, as well as immunoreactivity with anti-phospho-TP, indicating that PP2A was able to dephosphorylate MgcRacGAP in vitro at all Aurora B and Cdk1 target sites.

Since phosphorylation of MgcRacGAP by Cdk1 has not been previously described, we set to identify the phosphorylation site(s). Tandem mass spectrometric analysis of in vitro phosphorylated GST-GAP revealed that threonine 588, identified within a peptide ^{472}VSLLGPVTTPEHQLLKpT^{588}PSSSSLSQR, was the only residue phosphorylated by Cdk1. To confirm this finding, mutations were introduced in GST-GAP not only at position T588, but also at positions T567, T580 and T606 which, although

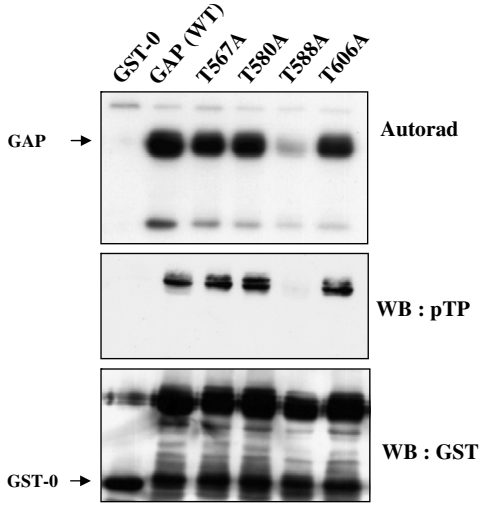

Fig. 6. Cdk1 phosphorylation of threonine residue 588. Following identification of pT588 by mass spectrometry, T to A mutations were introduced at positions 567, 580, 588 and 606 in GST-GAP. The resulting proteins were phosphorylated in vitro with Cdk1 as in Fig. 5 and analyzed by autoradiography, anti-phospho-TP Western blot and anti-GST as a loading control.

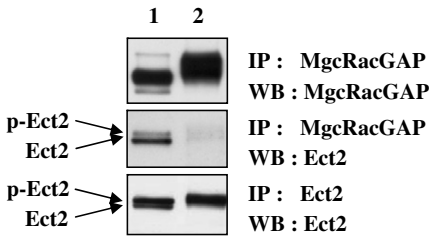

Fig. 7. Cell treatment with okadaic acid prevents Ect2 association with MgcRacGAP during mitosis. Cell lysates were prepared from cells arrested in prometaphase with nocodazole (lane 1), or nocodazole-arrested cells treated with okadaic acid (lane 2). MgcRacGAP was immunoprecipitated from the above lysates and membranes were probed with anti-MgcRacGAP (top) and anti-Ect2 antibodies (middle). Bottom panel shows Ect2 immunoprecipitates from the same lysates.

found in a non-phosphorylated state in our mass spectrometric analysis, were predicted as possible Cdk1 target sites by the Scansite software (http://scansite.mit.edu/, [26]). As shown in Fig. 6, converting T588 to A resulted in complete abrogation of Cdk1-mediated phosphate transfer to GST-GAP, as well as of GST-GAP reactivity with the anti-phospho-TP antibody, whereas mutations introduced at any of the other threonine residues had no effect.

3.5. Phosphoregulation of MgcRacGAP/Ect2 interaction

As mentioned previously, MgcRacGAP was found predominantly phosphorylated in prometaphase-arrested T lymphocytes. In the same cells, Ect2 was detected as a doublet which likely corresponds to Ect2 and phosphoEct2 (Fig. 7). The fast migrating unphosphorylated form of Ect2 coprecipitated with MgcRacGAP while only a minor amount of phosphoEct2 was present in MgcRacGAP immune complex. This result suggests that MgcRacGAP associates readily with Ect2 but poorly with phosphoEct2, as recently described by others [16].

When immunoprecipitated from okadaic acid-pretreated cells, MgcRacGAP migrated as a supershifted band, immunoreactive with anti-phospho-TP (Fig. 4). Of note, Ect2 was also hyperphosphorylated in the presence of OA indicating that Ect2 may be a physiological substrate of PP2A. Interestingly, OA treatment completely abrogated association of the two proteins, suggesting that PP2A mediated dephosphorylation of either MgcRacGAP or Ect2 plays a role in regulating MgcRacGAP/Ect2 interaction.

In conclusion, PP2A appears as a novel player in the phosphoregulation of MgcRacGAP and its interacting partners. Indeed, multiple Aurora B and Cdk1 mediated phosphorylation events are regulators of MKLP1/MgcRacGAP/Ect2 interactions and RhoGTPase activation throughout the progression of mitotic cells to cytokinesis; therefore, the ability of PP2A to dephosphorylate these sites may be critical to cytokinesis regulation and its exact role should be addressed in future studies.

Acknowledgements: We are grateful to Drs. D. Virshup and T. Kitamura for providing us with B56 and full length MgcRacGAP plasmids, respectively. We also thank Dr. Zee-Fen Chang (National Taiwan University) for helpful discussions. This work was supported in part by INSERM and CNRS, by grants from Ligue Nationale contre le Cancer (équipe labellisée) and ANR (BLAN06-2_135169) to J. Bertoglio and by grants from the "Association pour la Recherche sur le Cancer" and ANR (ANR-05-MRAR- 033-02) to G. Gacon.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.12.036.

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